

A new approach to determine loading efficiency of Leu-enkephalin in poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan

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This study is focused on a new approach for the determination of Leu-enkephalin loading efficiency in poly(isobutylcyanoacrylate) nanoparticles coated with depolymerized and thiolated chitosan (Mw 30, 85 and 145 kDa). Nanoparticles were obtained by anionic emulsion polymerization. The aim of this work was to propose a reliable method for the determination of the loading efficiency of Leu-enkephalin in nanoparticles. The amount of Leu-enkephalin contained in nanoparticles was determined after separation of loaded Leu-enkephalin from the dispersing medium using a modern and fast ultrafiltration technique as alternative to ultracentrifugation. The amount of Leu-enkephalin entrapped in nanoparticles was determined after total dissolution of nanoparticles in DMSO. Finally, the method developed in the present work was applied to the determination of the loading efficiency of nanoparticles prepared by two methods. The method consisting in loading nanoparticles with Leu-enkephalin at the same time as the preparation of the nanoparticles yielded higher loading efficiency (from 85 to 92%) than the method based on the adsorption of Leu-enkephalin on preformed nanoparticles (from 62 to 65%).

Key words: Nanoparticles – Leu-enkephalin – Poly(isobutylcyanoacrylate) – Thiolated chitosan – Ultrafiltration – Ultracentrifugation – Drug loading.

Recently, a system composed of poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan was investigated for the oral delivery of peptides. They are core-shell nanoparticles which form by auto-assembly of amphiphilic copolymers composed of hydrophobic poly(isobutylcyanoacrylate) moiety and hydrophilic part including thiolated chitosan with various molecular weights. Thiol groups confer (i) mucoadhesion by covalent attachment of thiol residues on glycoproteins of the mucus gel layer [1-3] (ii) permeation-enhancing properties presumably by interfering with the mechanism of glutathione regeneration (iii) and antiprotease activity due to their ability to bind divalent cations such as Ca²⁺, which are cofactors of many proteases [4]. These characteristics meant that poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan appeared to be promising materials for improving the oral delivery of peptides. Thus, the aim of the present work was to develop a relevant method for determining the association of Leu-enkephalin in these nanoparticles. Leu-enkephalin (Figure 1) was chosen here as a model peptide having an interesting morphinomimetic pharmacological activity applied in treatment against pain [5]. It is one of the relevant peptides to be administered by the oral route [6].

In the development of nanoparticles, one remaining critical step is the determination of the drug loading efficiency of the drug carrier. In general, this parameter is deduced from the determination of the non-associated fraction of the drug which can easily be measured in the dispersing medium after separation of the nanoparticles. Direct determination of encapsulated drug in nanoparticles is rarely proposed because it is more difficult to achieve in practice. Two obstacles may explain this fact. Firstly, nanoparticles must be totally dissolved to release the associated drug and secondly, the drug should be fully stable under the dissolution conditions of the nanoparticles.

Straightforward determination of the drug loading efficiency would provide important additional information about the availability of the drug after it was associated with the drug carrier. Indeed, part of the drug may be destroyed or irreversibly associated with the drug carrier during the preparation of the loaded nanoparticles and will not be further available for therapeutic action.

Developing a method that enabled the amount of peptide associated with poly(isobutylcyanoacrylate) nanoparticles coated with chitosan and thiolated chitosan to be directly determined by performing a modern and rapid method of nanoparticle separation based on ultra-filtration was explored and compared with those of the more generally used ultracentrifugation technique. Conditions to achieve complete dissolution of the nanoparticles were optimized and it was verified that the peptide remained stable during this critical step of the process. Finally, the developed method was applied to the determination of the drug-loading efficiency of Leu-enkephalin which was associated with the nanoparticles by two different drug loading methods.

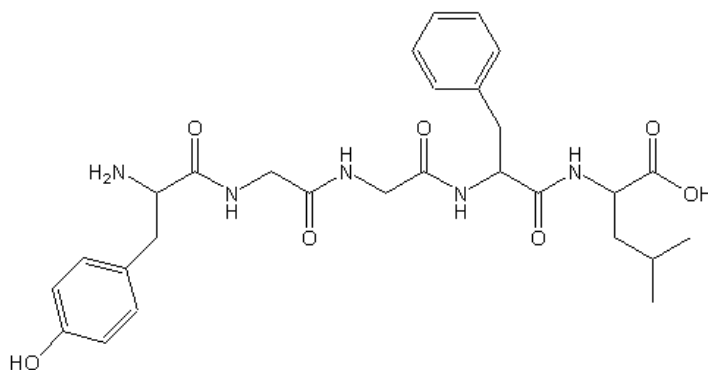


Figure 1 - Chemical formula of Leu-enkephalin.

I. Materials and Methods

1. Materials

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Chitosan Mw 400,000 g/mol, L-cysteine HCl, nitric acid, sodium hydroxide, dimethylsulfoxide (DMSO) and Leu-enkephalin (Mw 555 g/mol) were purchased from Fluka (Saint-Quentin-Fallavier, France). 2-iminothiolane HCl (Traut's reagent) was synthesized in the Department of Organic Chemistry (Biocis UMR CNRS 8076, Faculté de Pharmacie, Université Paris-11, Châtenay-Malabry, France). All chemicals were of analytical grade and used as received.

2. Methods

2.1. Chitosan depolymerization and characterization

Chitosan was selectively hydrolyzed following the method developed by Huang *et al.* [7]. The hydrolysis was performed using reaction with sodium nitrite at concentrations of 7, 2.7 and 1.6 g/L. The average molecular weights of the depolymerized chitosan were: 30,000 g/mol (Chito30), 85,000 g/mol (Chito85) and 145,000 g/mol (Chito145), respectively, as evaluated by capillary viscosimetry (viscosimeter AVS400, Schott Geräte). The percentages of deacetylation of chitosan were 60, 71 and 89% for Chito30, Chito85 and Chito145, respectively, as determined by ¹H-NMR analysis (Bruker MSL-400 spectrometer, Bruker Instrument Inc., Wissembourg, France) according to the method of Hirai *et al.* [8].

The inclusion of thiol groups in the hydrolyzed chitosan was carried out following the method developed by Bernkop-Schnürch *et al.* [1, 4]. Thiolated polymers were chitosan-4-thiol-butylamidine, named Chito30-TBA Chito85-TBA and Chito145-TBA according to the original molecular weight of the unmodified polymer.

2.2. Nanoparticle preparation

Nanoparticles were prepared by anionic emulsion polymerization according to the method of Bertholon *et al.* [9]. Briefly, 0.069 g of mixtures of hydrolyzed and thiolated chitosan (Chito/Chito-TBA 75/25% w/w) was dissolved in 5 mL of nitric acid in MilliQ water (0.2 mol/L), in a glass tube at 40°C, under gentle stirring and argon bubbling. After 10 min, 0.250 mL of IBCA were added under vigorous magnetic stirring. Argon bubbling was kept for additional 10 min and stopped. The reaction was allowed to continue at 40°C under gentle stirring for 50 min. After cooling to room temperature, pH was adjusted to 6.5 with NaOH (1 mol/L).

The purification of nanoparticles was achieved by dialysis using a Spectra/Por membrane with a molecular weight cut off of 100,000 g/mol (Biovalley, Marne-la-Vallée, France) twice 90 min and once overnight against 1 L of acetic acid 16 µmol/L. Control PIBCA nanoparticles were elaborated according to the same protocol except that chitosan and thiolated chitosan were replaced with 1% Pluronic F68).

2.3. Preparation of Leu-enkephalin-loaded nanoparticles

Two methods were tested to load nanoparticles with Leu-enkephalin: - in the "inclusion method", Leu-enkephalin was associated with the poly(isobutylcyanoacrylate) nanoparticles during their preparation. A small modification of the protocol of the preparation of the nanoparticles described above was introduced. A solution of Leu-enkephalin (0.5 mL at 1 mg/mL) was added to the polymerization medium just before the addition of IBCA. At the end of the preparation, the nanoparticles were not purified by dialysis;

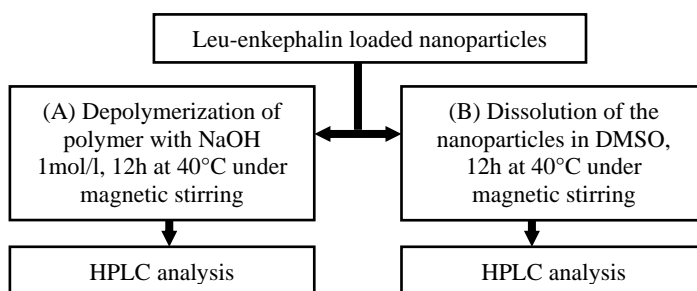


Figure 2 - Different strategies explored for the solubilization of PIBCA/Chito-TBA nanoparticles [9].

- in the "adsorption method", Leu-enkephalin was associated by adsorption on preformed poly(isobutylcyanoacrylate) nanoparticles. The nanoparticles were prepared and purified as described above. A solution (0.5 mL) of Leu-enkephalin solution (1 mg/mL) was then added to 5 mL of the nanoparticle suspension.

2.4. Determining the total amount of Leu-enkephalin associated with the nanoparticles

Nanoparticles were solubilized according to the methods described by Betholon *et al.* [9] (Figure 2). In the first method (method A), 5 mL of a solution of NaOH (1 mol/L) were added to 1 mL of nanoparticle suspension and was allowed to depolymerize the PIBCA part of the copolymer of the nanoparticles for 12 h at room temperature.

In the second method (method B), 5 mL of DMSO were added to 1 mL of nanoparticle suspension. The nanoparticles were allowed to dissolve at 40°C for 12 h. Experiments were performed in triplicate. The concentration of Leu-enkephalin in the samples was determined by high performance liquid chromatography (HPLC).

2.5. Investigation of the stability of Leu-enkephalin under the different experimental conditions

Firstly, the stability of Leu-enkephalin was studied in the acidic polymerization medium. A solution of Leu-enkephalin (0.5 mL at 1 mg/mL) was added to 6 mL of nitric acid (0.2 mol/L). The resulting solution was stirred for 1h at 40°C to mimic the polymerization conditions. The solution was stored one week at room temperature (25°C) before analysis.

Secondly, the stability of Leu-enkephalin was studied under the conditions of nanoparticle storage. A solution of Leu-enkephalin (0.5 mL at 1 mg/mL) was added to 6 mL of an aqueous solution at a pH of 6.5 adjusted with nitric acid and NaOH (1 mol/L). The solution was stored one week at room temperature (25°C) before analysis.

Finally, the stability of Leu-enkephalin was investigated under the conditions of nanoparticle hydrolysis and solubilization. A solution of Leu-enkephalin (0.5 mL at 1 mg/mL) was added to 6 mL of aqueous solution of NaOH (1 mol/L) for 12h at 40°C to mimic hydrolysis conditions. The same experiment was conducted by adding a solution of Leu-enkephalin (0.5 mL at 1 mg/mL) to 6 mL of DMSO for 12 h at 40°C to mimic nanoparticle solubilization conditions. At the end of each stability experiment, the concentration of Leu-enkephalin remaining in the samples was determined by HPLC. A 76-µg/mL aqueous solution of Leu-enkephalin served as a reference. All experiments were performed in triplicate from which the average and standard deviation were calculated. The percentage of lost Leu-enkephalin in the sample ($Leu-Enk_{lost}$) was calculated using Eq1 from the percentage of Leu-enkephalin found after incubation in the different conditions ($Leu-Enk_{after\ incubation}$):

$$Leu-Enk_{lost}(\%) = 100 - Leu-Enk_{after\ incubation}(\%) \quad Eq.1$$

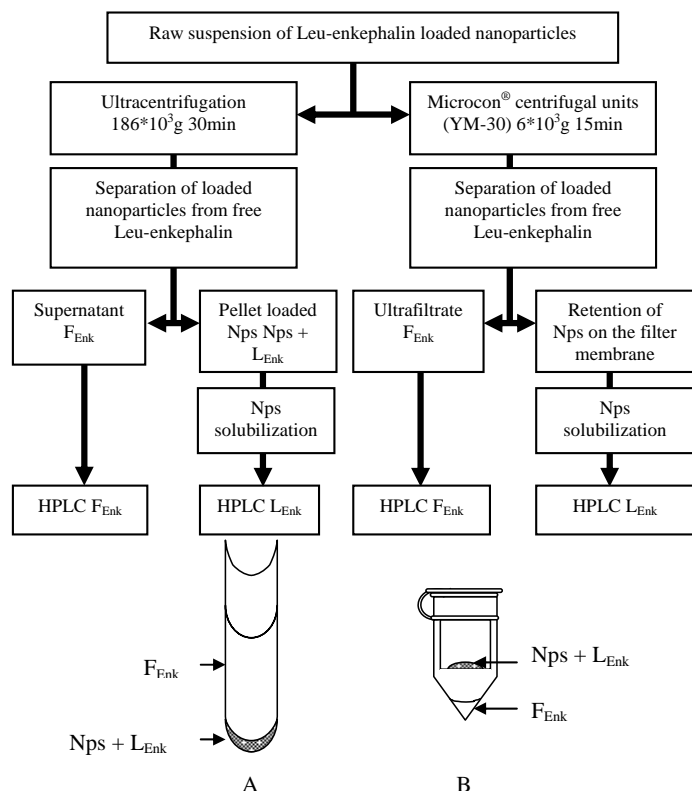


Figure 3 - Separation of Leu-enkephalin loaded nanoparticles from free Leu-enkephalin using ultracentrifugation (A) or ultrafiltration units (B). Loaded Leu-enkephalin: L_{Enk} , concentration of Leu-enkephalin associated with the nanoparticles. Free Leu-enkephalin: F_{Enk} , concentration of Leu-enkephalin remaining free in the dispersing medium of the nanoparticles.

2.6. Separation of Leu-enkephalin loaded nanoparticles from free Leu-enkephalin

Nanoparticles were separated from the dispersing media either by ultracentrifugation (30 min, 186×10^3 g, Optima Ultracentrifuge, Beckman-Coulter, Villepinte, France) or by ultrafiltration over Microcon centrifugal units YM-30 (Cut off 30,000) (15 min, 6×10^3 g) (Figure 3).

2.7. Physicochemical characterization of the nanoparticles

2.7.1. Particle size distribution

The hydrodynamic diameter of the nanoparticles and the size distribution were determined at 25°C by quasi-elastic light scattering using a Nanosizer N4 PLUS (Beckman-Coulter, Villepinte, France). The scattered angle was fixed at 90° and 60 μ L of each sample was diluted in 2 mL of MilliQ water.

2.7.2. Zeta potential determination

Zeta potential of nanoparticles was measured using Zetasizer Nanoseries (Malvern Instruments Ltd. UK). Dilution of the suspensions (1:33 (v/v)) was performed in NaCl (1 mmol/L).

2.8. Determination of Leu-enkephalin concentration

Samples containing Leu-enkephalin were analyzed by reversed phase HPLC using a Waters system equipped with a symmetry C18 column (4.5-150 mm) and UV detection at 220 nm. The mobile phase was a 32 min gradient mixture of (A) acetonitrile/trifluoroacetic acid 0.1% in water (10:90) and (B) acetonitrile/trifluoroacetic acid 0.1% in water (90:10). These HPLC analyses were performed at room temperature at a flow rate of 1 mL/min. The validated range of concentrations was 0.5-23.33 μ g/mL. Relative standard deviations were below 5% and recovery was always within the 95-105% interval.

2.9. Determining the yield of Leu-enkephalin loading

The yield of Leu-enkephalin loading (Y), expressed as a percentage, was calculated according to Equation 2:

$$Y (\%) = (L_{Enk}/T_{Enk}) 100 = [(T_{Enk} - F_{Enk})/T_{Enk}] 100 \quad \text{Eq.2}$$

where L_{Enk} is the loaded Leu-enkephalin (concentration of Leu-enkephalin associated with the nanoparticles), F_{Enk} the free Leu-enkephalin (concentration of Leu-enkephalin found in the dispersing medium after separation of the nanoparticles), and T_{Enk} the total Leu-enkephalin (concentration of Leu-enkephalin recovered from the total nanoparticle suspension). In order to be comparable, all concentrations were reported to the same volume for each sample. Free unloaded Leu-enkephalin contained in the dispersing medium was isolated from Leu-enkephalin loaded nanoparticle suspension by ultracentrifugation or ultrafiltration over Microcon centrifugal units YM-30 (30,000 MWCO) as described in section I.2.6 (Figure 3). The concentration of Leu-enkephalin associated with the nanoparticles and the total Leu-enkephalin concentration recovered in the nanoparticle suspension were obtained after 0.2 mL of nanoparticles were dissolved in 1 mL of DMSO at 40°C for 12 h.

II. Results and discussion

The preparation of nanoparticles by the emulsion polymerization of IBCA was achieved by the dispersion of the monomer in an acidic solution of chitosan/chitosan-TBA mixture. The polymerization of IBCA in nitric acid in the presence of chitosan produces copolymers of poly(isobutyrylcyanoacrylate) and chitosan which auto-associate to form nanoparticles composed of PIBCA-chitosan amphiphilic copolymers. Nanoparticle preparations in the presence of chitosan and thiolated chitosan were characterized by diameters varying from 135 to 371 nm (Table I). The zeta potential of PIBCA nanoparticles coated with thiolated chitosan was positive in agreement with the charge of the chitosan. The characteristics of control nanoparticles prepared with Pluronic F68 complied with those found in the literature [4, 10].

Table I - Mean hydrodynamic diameter (D_M) and ζ potential of PIBCA nanoparticles coated with Chito/Chito-TBA (75/25% w/w) and control nanoparticles elaborated by anionic emulsion polymerization of Leu-enkephalin remaining free in the dispersing medium of the nanoparticles.

Formulations	D_M (nm)	ζ potential (mV)
PIBCA/Chito 30-TBA ^a	135	+38 ^a
PIBCA/Chito 85-TBA ^a	230	+42 ^a
PIBCA/Chito 145-TBA ^a	371	+53 ^a
Control nanoparticles with Pluronic F68 ^b	160	-17.7* / -7.5**

*. pH =6.5, **. pH =1.7

1. Investigation of the stability of Leu-enkephalin under the different experimental conditions used in the present study

This part of the study was aimed at ensuring that Leu-enkephalin remained intact after nanoparticle preparation and the various conditions of treatments designed to dissolve the nanoparticles. Leu-enkephalin was incubated into two different media, at pH 1.2 to mimic the acidic medium of the polymerization and at pH 6.5 to investigate the stability in the storage medium. Results in Table II showed that Leu-enkephalin was stable in both media since 98-100% of the drug was recovered in the media by HPLC analysis performed after incubation (Table II). The stability of Leu-enkephalin was also investigated in the medium used to solubilize the nanoparticles which were used to release the associated drug. Indeed, both heat and basic treatment can denature peptides like Leu-enkephalin. The results of this study are presented in Table III. As indicated by the percentage of intact Leu-enkephalin recovered after incubation in each medium, Leu-enkephalin was significantly destroyed during the incubation under the basic conditions (method A). In contrast, Leu-enkephalin resisted to an incubation in DMSO at 40°C for 12 h (Figure 4) (method B).

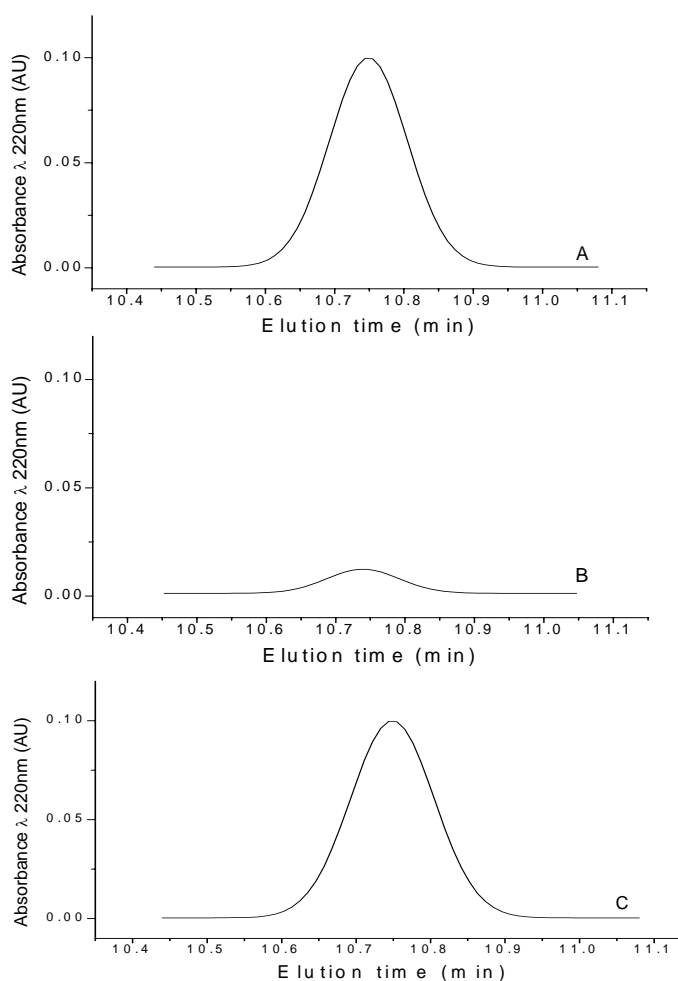
Table II - Stability of Leu-enkephalin determined after one week of incubation at 25°C in the polymerization medium and in the storage conditions (n = 3).

Stability medium	Theoretical concentration of Leu-enk (µg/ml)	[Leu-enk] before incubation (µg/ml)	[Leu-enk] after incubation (µg/ml)	% Leu-enk recovered after incubation	% Lost Leu-enk during incubation*
pH 1.2	75.9	75.9 ± 0.9	74.8 ± 0.9	98.6	1.4
pH 6.5	75.8	75.7 ± 0.7	74.8 ± 1.0	98.8	1.2
MilliQ® water	76.0	75.9 ± 0.8	75.9 ± 0.7	100	0

*. Calculation: $\text{Leu-enk}_{\text{lost}} (\%) = 100 - \text{Leu-enk}_{\text{after incubation}} (\%)$

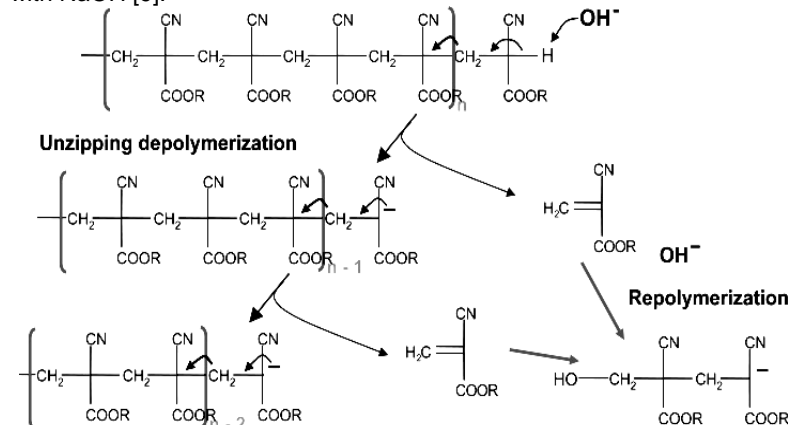
Table III - Stability of Leu-enkephalin determined in the conditions of dissolution of the nanoparticles. Samples were incubated for 12 h at 40°C in different media (n = 3).

Medium of incubation	Samples		[Leu-enk] before incubation (µg/ml)	[Leu-enk] after incubation (µg/ml)	% Leu-enk recovered after incubation	Lost Leu-enk (%) ^b
	Nanoparticles ^a	Leu-enk solution (µg/ml)				
DMSO	PIBCA/ Chito30-TBA	74.8	74.0 ± 1.0	73.0 ± 1.0	98.7	1.3
	PIBCA/ Chito85-TBA	68.9	68.0 ± 1.1	66.3 ± 1.1	96.1	3.9
	PIBCA/ Chito145-TBA	73.5	73.0 ± 1.2	72.5 ± 1.2	98.0	2.0
	-	75.9	75.8 ± 1.0	74.8 ± 0.9	98.8	1.2
NaOH 1mol/l	PIBCA/ Chito30-TBA	75.5	75.2 ± 1.0	26.4 ± 1.0	35.1	64.9
	PIBCA/ Chito85-TBA	72.0	72.0 ± 1.1	26.3 ± 1.1	36.6	63.4
	PIBCA/ Chito145-TBA	73.5	73.5 ± 1.2	25.2 ± 1.2	34.3	65.7
	-	75.8	75.7 ± 0.9	20.2 ± 0.2	26.8	73.2
MilliQ® water	-	76.0	75.9 ± 0.8	75.9 ± 0.7	100	0

**Figure 4** - HPLC Chromatograms of Leu-enkephalin obtained after incubation in DMSO (A), in NaOH (1 mol/L) (B) and in MilliQ® water (control experiment) (C) for 12 h at 40°C. The initial concentration of Leu-enkephalin in each sample was 76 µg/ml.

2. Recovery of Leu-enkephalin from a dispersion of Leu-enkephalin-loaded nanoparticles

Solubilization of chitosan-coated PIBCA nanoparticles is not easy to achieve because they are composed of a poly(alkylcyanoacrylate)-polysaccharide copolymer which is particularly difficult to dissolve. Bertholon *et al.* [9] have succeeded in dissolving similar nanoparticles composed of dextran-PIBCA copolymer either in hot DMSO or after partial hydrolysis of the PIBCA part of the copolymer using sodium hydroxide (Figure 5). The dissolution procedure using hot DMSO applied to Leu-enkephalin loaded nanoparticles led to recovery ranging from 96% to 99% of the initial amount of Leu-enkephalin, depending on the type of chitosan used for preparing the nanoparticles (Table III). This result indicated that this dissolution method did not denature or destroy Leu-enkephalin and it also indicated that Leu-enkephalin can be released from nanoparticles as intact peptide and quantitatively regarding the initial amount introduced in the preparation medium of the loaded nanoparticles.

Figure 5 - Scheme of the depolymerization of the PIBCA by reaction with NaOH [9].

3. Separation of Leu-enkephalin loaded nanoparticles from free Leu-enkephalin

Usually, drug loaded nanoparticles are isolated from the free drug remaining in the aqueous medium using ultracentrifugation [11-14]. Here, the use of ultrafiltration units was explored as an alternative method to ultracentrifugation. Results given in *Table IV* showed no significant differences when using ultracentrifugation or Microcon centrifugal units. Since the results obtained using the two techniques were quite similar, the separation of nanoparticles using ultrafiltration with Microcon centrifugal units was selected. Indeed, this technique is fast, easy to handle and does not require any sophisticated apparatus in comparison with ultracentrifugation. In addition, it is perfectly adapted for small samples (0.5-1 mL) while the ultracentrifugation required greater volumes of sample. In order to validate the ultrafiltration methodology from an analytical point of view, the possible adsorption of the peptide at the surface of the filters was checked by determining the concentration of Leu-enkephalin before and after centrifugation under the conditions described above. Results showed no adsorption of Leu-enkephalin to the Microcon centrifugal units since ($99 \pm 2\%$) of the peptide was recovered in the ultrafiltrate at the end of the process.

4. Determining Leu-enkephalin loading efficiency on nanoparticles obtained by two loading methods

The loading of the nanoparticles with Leu-enkephalin was determined using the new methodological approaches developed above. The loading of the nanoparticles was explored by adding the peptide either in the polymerization medium or after preparation of the nanoparticles. In the first case, it should be mentioned that the yield of association of Leu-enkephalin with nanoparticles was determined on the raw nanoparticle suspension.

We chose to skip the dialysis to avoid any artefact due to premature release of the drug during purification of the nanoparticles by dialysis. The yields of encapsulation determined the nanoparticles, Chito30-TBA, Chito85-TBA and Chito145-TBA (*Table V*). Alternatively, when expressed in mass the corresponding Leu-enkephalin associated amounts were 1.22, 1.23 and 1.27 mg/g of polymer. As a common problem encountered when the polymer forming the nanoparticles is synthesized in the presence of the drug, it is noteworthy that the total Leu-enkephalin recovered after the dissolution of the nanoparticles (T_{Enk}) was close to the initial amount of Leu-enkephalin incorporated in the polymerization medium, suggesting the absence of any covalent attachment of the peptide to the polymer.

Adsorption of Leu-enkephalin on previously prepared nanoparticles was explored as an alternative method of drug loading. This method presents the advantage that it can be performed on purified nanoparticles, which suspensions were cleared from residual reagents of the polymerization. The results summarized in *table V* indicated that relatively high drug loading efficiency can be obtained by adsorption of Leu-enkephalin on the Chito-TBA coated nanoparticles. The yields of association were 61.8, 62.9 and 65.0% depending on the coating of the nanoparticles, Chito30-TBA, Chito85-TBA and Chito145-TBA (*Table V*). Alternatively, when expressed in mass the corresponding Leu-enkephalin associated amounts were 0.88, 0.89 and 0.90 mg/g of polymer. It can be postulated that adsorption of Leu-enkephalin can occur via ion pair formation with cationic chitosan. Indeed, the isoelectric point of Leu-enkephalin is 5.8, which implies that the peptide is negatively charged at pH 6.5 while chitosan and thiolated chitosan are positively charged in the storage medium in which the association by adsorption was performed.

Table IV - Optimization of the separation process of loaded nanoparticles from the aqueous medium using ultracentrifugation at 186×10^3 g during 30 min and Microcon centrifugal units at 6×10^3 g during 15 min ($n = 3$). The concentration of Leu-enkephalin added in the polymerization medium for the preparation of Leu-enkephalin loaded nanoparticles was 76 μ g/mL

Formulation	Ultracentrifugation				Microcon [®] centrifugal units			
	F _{Enk} (μ g/ml)	L _{Enk} (μ g/ml)	T _{Enk} (μ g/ml)	Loading Y (%)	F _{Enk} (μ g/ml)	L _{Enk} (μ g/ml)	T _{Enk} (μ g/ml)	Loading Y (%)
PIBCA/ Chito30-TBA	11.6 \pm 0.4	64.4 \pm 0.8	76.0 \pm 1.2	84.8	11.3 \pm 0.5	64.3 \pm 0.9	75.6 \pm 1.0	85.0
PIBCA/ Chito85-TBA	10.7 \pm 0.4	65.0 \pm 0.7	75.7 \pm 1.0	85.9	10.8 \pm 0.5	65.2 \pm 0.8	76.0 \pm 1.1	85.8
PIBCA/ Chito145-TBA	6.9 \pm 0.5	69.0 \pm 0.7	75.9 \pm 0.9	90.9	65.0 \pm 0.4	69.2 \pm 0.8	75.7 \pm 1.2	91.4

L_{Enk}: concentration of Leu-enkephalin associated with the nanoparticles. F_{Enk}: concentration of Leu-enkephalin found in the dispersing medium after separation of the nanoparticles. T_{Enk}: concentration of Leu-enkephalin recovered from the total nanoparticle suspension.

Table V - Comparison of the Leu-enkephalin associated with nanoparticles obtained by incorporation of Leu-enkephalin (76 μ g/mL) in the polymerization medium prior to the formation of nanoparticles or by adsorption on already prepared nanoparticles.

Method of Leu-enkephalin association	Leu-enkephalin added to the polymerization medium				Adsorption of Leu-enkephalin on already prepared nanoparticles			
	F _{Enk} (μ g/ml)	L _{Enk} (μ g/ml)	T _{Enk} (μ g/ml)	Loading Y (%)	F _{Enk} (μ g/ml)	L _{Enk} (μ g/ml)	T _{Enk} (μ g/ml)	Adsorption Y (%)
PIBCA/Chito30-TBA	11.3 \pm 0.5	64.3 \pm 0.9	75.6 \pm 1.0	85.0	28.8 \pm 0.6	46.6 \pm 0.7	75.4 \pm 0.9	61.8
PIBCA/Chito 85-TBA	10.8 \pm 0.5	65.2 \pm 0.8	76.0 \pm 1.1	85.8	28.2 \pm 0.7	47.7 \pm 0.8	75.9 \pm 1.0	62.9
PIBCA/Chito 145-TBA	65.0 \pm 0.4	69.2 \pm 0.8	75.7 \pm 1.2	91.4	26.5 \pm 0.5	49.1 \pm 0.7	75.6 \pm 1.1	65.0

L_{Enk}: concentration of Leu-enkephalin associated with the nanoparticles. F_{Enk}: concentration of Leu-enkephalin found in the dispersing medium after separation of the nanoparticles. T_{Enk}: concentration of Leu-enkephalin recovered from the total nanoparticle suspension.

Conclusion

In this work we proposed a step by step methodology to investigate the loading of chitosan-coated PIBCA nanoparticles using Leu-enkephalin as a model peptide. The methods included the determination of the total amount of intact peptide remaining in the suspension and available as a therapeutically active form. It also provided a determination of the drug loading efficiency performed directly on the nanoparticles. These determinations were made possible by the complete dissolution of the nanoparticles under conditions in which the peptide was found to remain stable. In this study, it was also shown that ultrafiltration using Microcon units is a suitable separation method of nanoparticles from the dispersion medium which is as efficient as the ultracentrifugation but presents the main advantages of being much faster and applicable on very small samples.

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